REMARKS

CLAIM STATUS

Claims 29-33 are currently pending in the application.

Rejection Under 35 U.S.C. §103

Claims 29-33 have been rejected under 35 U.S.C. 103(a) as being unpatentable over Columbo et al. (J. Immunol. 160:2780-2785 (1998)) in view of Pauli et al. (Clinical and Experimental Allergy, 30:1076-1084 (2000)).

The Examiner contends that Columbo et al. teach Parj 1 and Parj 2 are the two major allergens in Parietaria judaica pollen, and teach that mutation of positions K21, K23, E24 and K27 with alanine leads to a loss of IgE binding. The Examiner contends that Pauli et al. teach dimer and trimer multimer proteins of Bet vI in pharmaceutical compositions exhibited reduced skin reactions. The Examiner concludes that it would have been obvious to one of ordinary skill in the art at the time of invention to combine the teachings of Columbo et al. and Pauli et al. to produce a multimer protein comprising Parj 1 and Parj 2 to treat allergies. The rejection is respectfully traversed.

Applicants submit that Columbo et al. only focuses on Parj 1 and the identification of putative IgE binding regions. In particular, Colombo et al. describe the IgE binding activity of a portion of the Parj1 allergen (i.e. the region 1-30). In this context, Colombo et al. teach:

- that the cysteines in position 14 and 29 are essential for IgE binding (*see* Colombo et al., paragraph "epitope mapping" from p. 2781 to p. 2782); and

- that single mutation K21, K23, E24 or K27 causes loss of binding (*see* p. 2782, left column, second paragraph, Fig. 3A and 3B.

Applicants submit that Columbo et al.

- do not report the effect of a single mutation (K21, or K23 or E24 or K27) on the full length Parj 1;
- do not report the effect of more than one mutation on the 30 amino acids derived Parj 1 molecule or on the full length Parj 1 molecule; and
- do not report any data on Parj 2 and its mutations.

Regarding Pauli et al, Applicants submit that Pauli indicates in the introduction at p. 1077, left column, first sentence that there are two birch pollen allergens, Bet v1 and Bet v2. Pauli et al., however, only describe the allergenic activity of birch pollen allergen Bet v1. Pauli teaches oligomers (dimer and trimer) of Bet v1 that are produced by ligation of two or three copies of the Betv1 cDNA in a plasmid followed by expression in *E. coli* (*see* Pauli at p. 1077, left column, third paragraph).

The homodimer (Betv1-Betv1) and homotrimer (Betv1-Betv1-Betv1) are shown to induce no reaction at a concentration of 10 ug/ml in the prick test (*see* Pauli at p. 1079, left column, first paragraph and Fig. 2). In addition, the Betv1 homotrimer seems hypoallergenic after intradermal testing of birch allergic patients (*see* Fig. 3b and Fig. 4a). Pauli indicates at p. 1081, right column, last sentence to p. 1082, left column, first sentence that: "*one has to consider that the absence of local*

reactions during intradermal testing cannot predict the absence of systemic effects during immunotherapy".

Thus, Applicants submit that that the teachings of Pauli are strictly limited to multimers of the same molecule and to Betv1 allergens. Pauli et al. do not teach any other strategy of cloning.

In contrast, the molecules of the present invention comprise a combination of a full length Parj 1 mutated in three specific positions, namely K23, E24 and K27, and a full length Parj 2 mutated in three specific positions, namely K23, E24 and K27.

<u>The combination of Columbo et al. and Pauli et al.</u>

Columbo relates only to single mutants of the 1-30 portion Par j 1. Based on the teaching of Columbo, cysteines replacement by serine at position 14 and 29 as well as mutation of the single residues K21, K23, E24 or K27 in a Parj 1 derived molecule of 30 amino acids would decrease or cause loss of IgE binding.

Therefore, starting from Columbo et al. with the aim of producing a potential agent to reduce allergenic response to Par j 1, the skilled in the art would produce a molecule of 30 amino acids (see Fig. 3A) having mutated cysteines in position 14 or 29 or molecules of 30 amino acids having the single mutation K21, K23, E24 or K27. Columbo et al. do not teach or provide any data to show a molecule comprising full length Parj 1 in which three specific residues, namely K23, E24 and K27, are mutated. Let alone that such molecule could have hypoallergenic properties.

The combination of Columbo and Pauli would not lead to the molecules of the present invention because Pauli et al. only teach multimers of the same molecule. There is absolutely no suggestion in Colombo to combine Par j 1 and Par j 2 to create an hypoallergenic molecule. Colombo does not show any data on Par j 2 mutants; nor does Colombo indicate or suggest that mutations of the corresponding single residues K21, K23, E24 or K27 in a Parj 2 derived molecule of 30 amino acids would decrease or cause loss of IgE binding.

Therefore the present invention's combination of SEQ ID No. 2 and SEQ ID No. 4 is not obvious over Columbo alone or in combination with Pauli since again, the only suggestion of Pauli is to combine several molecules of the same allergen, i.e. homo-dimers/trimers and not hetero-dimers/trimers as claimed herein.

As another line of evidence, Applicants would like to indicate that a paper by Costa et al. (*see* **Exhibit A**) demonstrated that the Parj2 allergen contains at least four independent IgE epitopes. Disruption of one epitope does not necessarily exclude the possibility that the remaining IgE epitopes will not trigger the target cells (as shown in Costa et. al., Allergy (2000)).

Accordingly, Applicants submit that the present invention of making a molecule possessing hypoallergenic properties by merging two independent allergens (Parj1 + Parj2), each of which is mutated in just one IgE epitope, is not obvious in view of prior publication such as Costa et al.

In view of the above remarks, Applicants respectfully request that the rejection of claims 29-33 under 35 U.S.C. 103(a) be withdrawn.

CONCLUSION

Applicants believe that all grounds of objections and rejections raised in the outstanding Office Action have been fully addressed, and the claims are in condition for allowance. Accordingly, Applicants respectfully request favorable action to be rendered by the Examiner.

If a telephone interview would be of assistance in advancing prosecution of the subject application, Applicants' undersigned attorney invites the Examiner to telephone him at the number provided below. No fee is deemed necessary in connection with the filing of this Communication. However, if any fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 50-1891.

Respectfully submitted,

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Exhibit A

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Original article

The IgE-binding epitopes of rPar j 2, a major allergen of *Parietaria judaica* pollen, are heterogeneously recognized among allergic subjects

Pollen allergens are multivalent proteins that cross-link IgE antibodies on mast or basophil cells, inducing secretion of biologic mediators, and resulting in various allergic symptoms. The IgE-binding regions of the *Parietaria judaica* (*Pj*) pollen major allergen rPar j 2 were investigated. Twenty-nine single sera from *Pj*-allergic subjects were tested by Western blot against five recombinant peptides. At least four putative IgE-binding epitopes were identified. The analysis of their diffusion suggested a heterogeneous IgE-binding response. In fact, 75% of the sera reacted with peptide 1–54, 48% with peptide 48–101, 24% with peptide 1–30, 7% with peptide 29–54, and none with peptide 48–76. These five peptides were analyzed with the histamine-release assay. Only peptide 48–101 was capable of inducing degranulation and release of histamine. These results suggest that the recombinant rPar j 2 allergen contains IgE epitopes that are heterogeneously recognized by sensitive patients, and that therefore the therapeutic approach based on the use of haptenic peptides needs a careful evaluation.

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Key words: allergen; epitope; IgE; *Parietaria judaica*; recombinant allergen.

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Type I hypersensitivity affects millions of people worldwide, and its incidence (20–30%) is increasing in most industrialized countries, leading to rising human and economic costs (1). Pollen allergens are usually glycoproteins that interact with IgE bound to $Fc_{\varepsilon}RI$ receptors on mast cells and basophils, leading to aggregation of the receptors with consequent release of mediators and specific allergic symptoms. The current therapy consists of injection of increasing doses of partially purified extracts of the allergenic source. The disadvantage of this approach is the risk of possible IgE-mediated anaphylactic side reactions due to the active allergens injected. A way to overcome this could be to identify on the allergen IgE-binding epitopes that bind and saturate the cytophilic IgE and block further interaction with native allergens without any IgE cross-linking reaction (2). It is therefore important to identify these haptenic epitopes and to ascertain the diffusion of their IgE interaction among the allergic subjects. The pollen of the weed *Parietaria judaica* (*Pj*) is the main cause of allergy in the Mediterranean area, affecting approximately 10 million people (3, 4). Recombinant DNA technology has proved to be a useful tool in the isolation and characterization of allergenic molecules from several sources (5–7), and two cDNA clones coding for two major allergens of *P. judaica* pollen, i.e., Par j 1 and Par j 2, have been isolated and the recombinant allergens characterized (8, 9). The three-dimensional structure of rPar j 1 was determined by structural homology modeling, and the data were used to identify putative IgE-binding epitopes (10). The same recombinant allergen was used to study the T-cell response (11, 18). In the present study, we selected the recombinant allergen rPar j 2 to establish the number and diffusion of IgE-binding epitopes among the *Pj*-allergic subjects and their immunologic properties. We found that this allergen contains at least four main IgE-binding epitopes, and that their presence among *Pj*-allergic people is quite heterogeneous.

Material and methods

Cloning in pMALC2 and cDNA sequencing

Different clones were obtained by PCR amplification. The oligonucleotides were flanked by the restriction enzyme EcoRI and XbaI sites. An amount of 1 ng of the Par j 2 clone was subjected to 30 cycles of the

* During the preparation of this paper, Roberta Cocchiara passed away, after a long battle with a severe form of rheumatoid arthritis, on 7 August 1998.

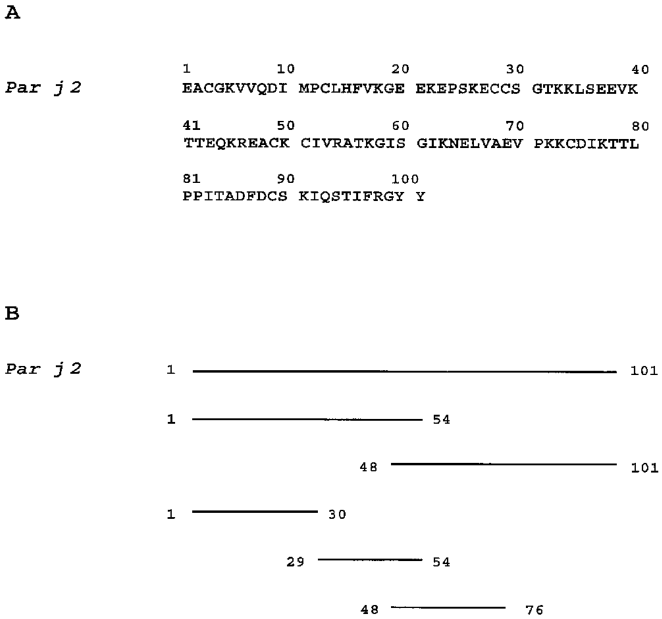

Figure 1. A) Deduced amino-acid sequence of recombinant Par j 2 (8); B) schematic representation of rPar j 2 peptides.

following conditions: 94°C for 30 min, 52°C for 30 min, and 72°C for 30 min. The PCR products were purified, digested with EcoRI and XbaI restriction enzymes, and cloned in frame in the EcoRI-XbaI sites of the pMALC2 vector (BioLabs, UK). All the clones were sequenced by the dideoxy chain termination method with a Sequenase Kit (Amersham, USA).

Preparation of recombinant proteins

The recombinant clones were grown at 37°C to a density of 0.5–0.6 OD_{600} in LB broth with the appropriate antibiotic and induced for 2 h with 0.3 mM isopropylthio-β-D-galactoside. The cells were harvested by centrifugation at 4000 *g* for 20 min, and the pellet was then dissolved in PBS (10 mM sodium phosphate, pH 7.2, 200 mM NaCl, 1 mM EDTA, and 1 mM NaN_3) and lysed by sonication with the Heat System Ultrasonic, W-385. The cell debris was removed by centrifugation at 9000 *g* for 30 min. The recombinant proteins were purified by affinity chromatography on an amylose resin column (BioLabs, UK). The concentration of the recombinant proteins was determined by densitometric analysis of SDS–PAGE stained with Coomassie brilliant blue and compared to a standard protein.

Immunoblot and inhibition

An amount of 2 μg of each recombinant protein was fractionated on 10% SDS–PAGE and electroblotted onto PVDF membrane (Immobilon P Millipore, USA). After blotting, membranes were incubated for 3 h with blocking buffer (PBS supplemented with 3% BSA, 0.5% Tween-20, and 0.02% NaN_3) and washed three times with PBS containing 0.1% Tween-20. Inhibition was carried out by adding increasing amounts of recombinant proteins (5, 10, and 50 μg/ml) to a serum obtained from a *Pj*-allergic patient, diluted 1:20 for 3 h at room temperature. The filters were then incubated overnight with the serum previously adsorbed. After washing, the filters were incubated for 45 min with horseradish peroxidase HRP-conjugated rabbit antihuman IgE (Sigma, St Louis, MO, USA). The final reaction was developed with an ECL detection system (Amersham, USA). The intensity of the signal was measured with a BioRad densitometer, model GS-670. The percentage of inhibition was calculated by the following formula:

$$\% = 100 \times (A–B)/A$$

where A represents the signal intensity without the inhibitor and B the signal intensity with the inhibitor.

Human sera

The 29 sera used were collected from subjects not previously hyposensitized, with a clear clinical history of seasonal allergy toward the *Pj* pollen and a high RAST class level (3+, 4+) of IgE specific for *Pj* allergens. The five control sera were obtained from subjects not allergic to the *Pj* pollen and with negative RAST determination (0–5% of radiolabeled antihuman IgE bound).

Histamine-release assay

Human basophils were obtained by venipuncture of atopic subjects allergic to the *Pj* pollen and sharing a RAST value of 4+. The rPar j 2 protein (1 μg/ml) was used as a positive control, while the histamine released after challenging with the recombinant peptides was determined as previously reported (12). The net histamine released in response to the peptides was expressed as a percentage of the total cellular histamine after subtraction of the amount of histamine spontaneously released in the absence of any stimulus.

Results

Two overlapping DNA fragments covering the complete allergen sequence and corresponding to the peptides 1–54 and 48–101 were subcloned in pMALC2 vector and expressed as proteins fused to maltose-binding protein. A pool of sera ($n = 29$) from subjects allergic to the *Pj* pollen and reacting with the recombinant rPar j 2 allergen and a pool of sera ($n = 5$) from nonallergic subjects were used to determine the IgE-binding capability of the two peptides by Western blot analysis. The two peptides showed a strong IgE-binding reaction; therefore, we decided to restrict their length to obtain a better definition of the IgE-binding epitopes. Three shorter DNA fragments named Par 2

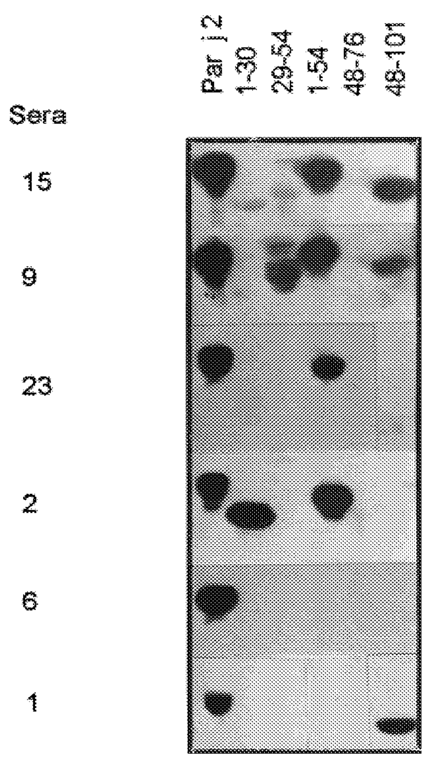

Figure 2. Western blot analysis. 2 μg of each recombinant protein was run on 10% SDS–PAGE and immunoblotted, as described in Material and methods. Specific IgE complexes were detected by HRP-conjugated rabbit antihuman IgE. Sera and recombinant proteins are shown on left and on top of blots, respectively.

Table 1. Signal intensity of recombinant proteins

Clones Sera	*Pj* 2	1–30	29–54	1–54	48–76	48–101
1	++					++
2	++	++		++		
3	++					
4	++			+		
5	++	++		++		
6	++					
7	++			±		
8	++					
9	++		++	++		+
10	++					+
11	++			++		±
12	++			+		+
13	++					
14	++			+		+
15	++	±		++		++
16	++			±		+
17	++			++		±
18	++			+		+
19	++			++		
20	++	±		++		±
21	++	±		++		++
22	++	+		++		+
23	++			++		
24	++			++		+
25	++					
26	++			+		
27	++		+	+		
28	++			++		
29	++	+		++		

Sera and recombinant proteins are shown on left and on top of table, respectively. Percentage of signal intensity was calculated by following formula:
$\% = 100 \times (A-B)/A$, where A represents signal intensity of Par j 2 and B signal intensity of recombinant proteins. ±: 10–30%; +: 30–60%; ++: 60–100%.

(1–30), Par 2 (29–54), and Par 2 (48–76) were subcloned into the pMALC2 vector and expressed as proteins fused to maltose-binding protein (Fig. 1). Sera from 29 *Pj*-allergic subjects reacting to the recombinant rPar j 2 allergen and used as a pool in the experiment described above were now used as a single serum in Western blot analysis with the five recombinant peptides already described. Four of these peptides showed a positive IgE-binding result (Fig. 2) that was heterogeneously distributed among the different sera analyzed (Table 1; Fig. 2). In fact, peptide 1–54 showed a positive IgE interaction with 75% of the sera tested (22/29) while peptide 48–101 was capable of IgE interaction with 48% of the sera (14/29). The other three shorter peptides showed a lower diffusion among the same sera; i.e., peptide 1–30 gave a positive IgE interaction with 24% of the sera analyzed (7/29), peptide 29–54 was positive with only 7% (2/29), and not one of the sera bound to peptide 48–76. In addition, 5/29 sera showed IgE against none of the five peptides used even though they showed positive IgE binding when the full-length rPar j 2 allergen was used as a substrate (Table 1). When the five sera obtained from nonallergic subjects were used as a control, the five peptides did not give any IgE-positive interaction (data not shown). Western blot inhibition was carried out to analyze further the contribution of the single peptide to the total IgE-binding value obtained with the full-length recombinant allergen. The two longer peptides covering the entire allergenic protein sequence were selected, and the results reported in Table 2 and Fig. 3 suggest a different involvement of the two regions.

These five peptides were therefore analyzed for their capability to induce histamine release by challenging basophils from six of the 29 subjects allergic to the *Pj* pollen used in the Western blot analysis; among the five peptides, only the 48–101 peptide showed reliable histamine-release activity (Table 3).

Discussion

The main problem of the immunotherapy used for type I allergy is the risk of anaphylactic reactions. One new approach to this problem is the use of molecules with no or reduced IgE-binding epitopes that are not capable of cross-linking $Fc_{\varepsilon}RI$ receptor-bound IgE on basophils or mast cells and therefore are subsequently not capable of inducing histamine release (13–16). Consequently, the goal is to determine the number and composition of the IgE-binding epitopes present on the allergenic molecule

Table 2. Inhibition of IgE binding by recombinant peptides

Sera	Proteins		
	rPar j 2	1–54	48–101
no. 23 added with rPar j 2 (50 μg/μl)	100	100	=
no. 23 added with 1–54	80	100	=
no. 15 added with 1–54	70	90	10
no. 15 added with 48–101	50	10	100
no. 15 added with 1–54 + 48–101	90	100	100
no. 15 added with rPar j 2	100	100	100

Values reported are expressed as percentage of inhibition calculated by formula % = 100 × (A–B)/A, where A is signal intensity without inhibitor, and B with inhibitor added.

in order to reduce or to avoid their IgE-binding properties by site-directed mutagenesis.

Different methods have been described in the literature and have been widely used to obtain information on the IgE-binding epitopes map of allergenic protein molecules (19–21). Probably the most widely used method was based on the analysis of the antigenic property of peptides obtained after enzymatic or chemical fragmentation of the allergenic protein. In view of several advantages offered by recombinant DNA technology, we have used the cDNA of the major allergen of *Pj* pollen, Par j 2, as a template in a PCR-based strategy, in order to localize the IgE-binding epitopes present on the recombinant rPar j 2 allergen. The first approach was made by using two long peptides and a pool of sera from subjects allergic to the *Pj* pollen. A strong IgE-binding activity for both the peptides was obtained, suggesting that restriction of the peptide length would increase the possibility of finer mapping of the IgE-binding epitopes

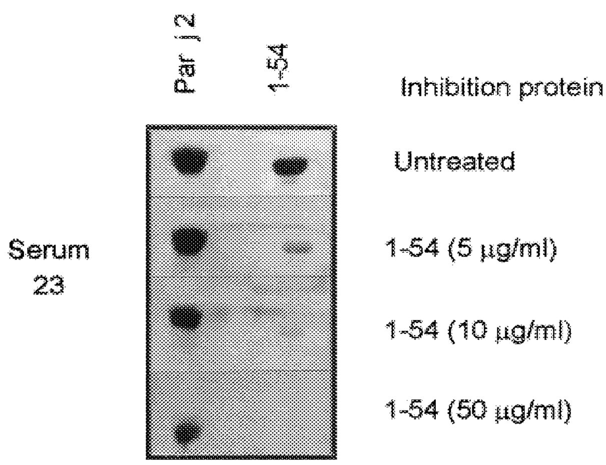

Figure 3. Western blot inhibition. Recombinant proteins separated on 10% SDS–PAGE were immunoblotted as described in Materials and methods. Sera were adsorbed for 3 h at room temperature with increasing amounts (5, 10, 50 μg/ml) of recombinant proteins. Specific IgE complexes were detected by HRP conjugated to rabbit antihuman IgE. Recombinant proteins are shown on top and single sera on left of blots.

(17). Therefore, by using three additional shorter peptides and single sera, we found that the recombinant rPar j 2 allergen contains at least four IgE-binding epitopes. The first epitope was found on the NH_2-terminal region 1–30, and considering the sequence homology shared by the two *Pj* major allergens Par j 1 and Par j 2, it is very likely that the epitope in the region 1–30 is similar to the epitope found in the same region of the rPar j 1 allergen already described (10). The second epitope has been localized in the region 29–54, while a third epitope is localized in the region 76–101 since the peptide 48–76 is not capable of binding IgE. A fourth epitope must be present in the region 48–101, since this peptide was capable of releasing histamine by bridging IgE on basophils. Looking at the distribution of these putative IgE-binding epitopes, we found that they were not equally recognized by the sera. Some of these peptides interacted with more than 70% of the sera and others with less than 10% of the same sera. One can conclude from this result that although peptides incorporating IgE-binding epitopes can be used as alternative reagents for successful immunotherapy, a preliminary and careful selection of the most appropriate IgE-binding epitopes must be carried out by using methods directed to quantitate what percentage of the IgE binding to the full-length allergenic protein is shared by the single peptide.

However, these data are probably not adequate to predict the efficacy of a peptide in immunotherapy since its activity as a histamine releaser must be preliminarily checked. A histamine-release assay carried out by testing the peptides suggested that only the 48–101 peptide was capable of inducing histamine release, and therefore it is to be avoided since it can induce a dangerous anaphylactic reaction.

In conclusion, the data reported in this study suggest that the four putative IgE-binding epitopes of the major

Table 3. Histamine-release activity of recombinant peptides

Subjects	Stimuli						
		Peptides					
	rPar j 2	1–54	48–101	1–30	29–54	48–76	Anti-IgE
1	51++	5	30++	2	4	4	51
2	33++	3++	3	4++	3	4	63
6	27++	4	9	4	5	6	34
9	81++	4++	56+	5	2++	3	42
15	70++	2++	33++	4±	6	3	54
23	25++	4++	3	6	5	4	7
D.G.	4	3	4	5	3	3	46

Concentration of stimuli was 1 μg/ml. Recombinant allergen rPar j 2 was used as positive control. D.G. was nonallergic subject. Values of histamine release reported are expressed as percentage calculated as follows:
% = (stimulus − spontaneous release)/(total release − spontaneous release) × 100.
IgE binding values − ±: 10–30%; +: 30–60%; ++: 60–100%.
Percentage of IgE binding was calculated by following formula:
% = 100 × (A–B)/A, where A represents signal intensity of Par j 2 and B signal intensity of recombinant proteins.

allergen Par j 2 are heterogeneously recognized among the subjects allergic to the *Pj* pollen, and that the use of peptides representing haptenic IgE-binding epitopes requires a preliminary analysis of the IgE specificity of the allergic subject.

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